

# Application of qPCR method for transgene copy number determination in recombinant CHO cells in biopharmaceutical industry

Škulj Mihaela, Okršlar Veronika, Jamnikar Uroš, Gaser Dominik, Matjaž Brinc, Francky Andrej

Biopharmaceuticals, Cell and Molecular Biology  
Lek Pharmaceuticals d.d.  
Kolodvorska 27, SI-1234 Mengeš

## Introduction

Cultivated mammalian cells have become the dominant system for the production of recombinant proteins for clinical applications because of their capacity for proper protein folding, assembly and post-translational modifications. CHO cells are most frequently used for protein production. Firstly cells are transfected with plasmid carrying a transgene and additionally a gene conferring a selective advantage. In the presence of the selection agent only those cells that express the selector gene survive. Following selection, survivors are cultivated as single clones. Clones are then evaluated and the cell line with the appropriate growth and productivity characteristics is chosen for the production of recombinant protein (1). It is very important that the cell line is stable during the production phase. The transgene sequence, its copy number, number of integration sites, length of mRNA transcript and other parameters are evaluated on the genetic level. Since determination of transgene copy number and genetic stability are among the important parameters for recombinant protein production, a fast and accurate method for its determination had to be developed. In this work a qPCR method for transgene copy number determination using absolute quantification and TaqMan chemistry was developed.

## Materials and methods

### Cell cultures

Recombinant CHO-K1 and CHO dhfr- cell lines of chinese hamster ovary (CHO) were used for recombinant protein production.

- CHO K1 cells were cotransfected with two plasmids one containing the sequences for the A and B subunit of the recombinant protein and the other containing a gene for antibiotic resistance. Plasmids were introduced into cells in ratio 10:1. After final clone selection a genetic stability test was performed. Cells were grown in 100ml spinner flasks for 120 generations and samples for copy number determination were collected every 20 generations (culture was transferred to 500 ml spinner flasks to obtain enough cells for genetic analysis).
- CHO dhfr- cells were used for testing of the influence of methotrexate on the transgene copy number. Cells were cotransfected with two plasmids, one containing a gene for DHFR and the other containing sequences for the A and B subunit of a recombinant protein. Cells were grown in T-flasks until confluency and passaged every two days. Cells were grown in 7 parallels and were treated with an increasing concentration of methotrexate, which was 0, 200, 400, 800, 1000, 1500, 2000 nM. During cell growth the concentration of MTX was slowly elevated to the final concentration. Production was measured using an ELISA test.

### qPCR

For copy number determination a qPCR method using absolute quantification was developed. Specific amplicons on chromosome and plasmids were constructed. The amplicon on chromosome was constructed on a single copy gene for glucagon (GLUC). On plasmids the amplicons were constructed on specific transgenes for the A and B subunit of the recombinant protein and on a gene for antibiotic resistance. For standard curve preparation, isolated genomic DNA from nontransfected cells and isolated plasmid DNA were used. Five serial dilutions were prepared in the range from approx. 100-10000 copies per well (100pg/μl-10ng/μl) for genomic DNA and from 500-5×10<sup>6</sup> copies per well (1fg/μl-10pg/μl) for plasmid DNA. After analysis results were evaluated. Dilutions where CV of three replicates was > 0,3 were not used for standard curve preparation and copy number determination. Genomic DNA from the sample was also prepared in five serial dilutions in the same range as for the standard curve of genomic DNA and relative standard curves were constructed for each sample and efficiency of amplification was calculated. Samples where efficiencies of amplification differed more than 10% compared to those of the standards were reanalyzed. The copy number was determined as a ratio between the

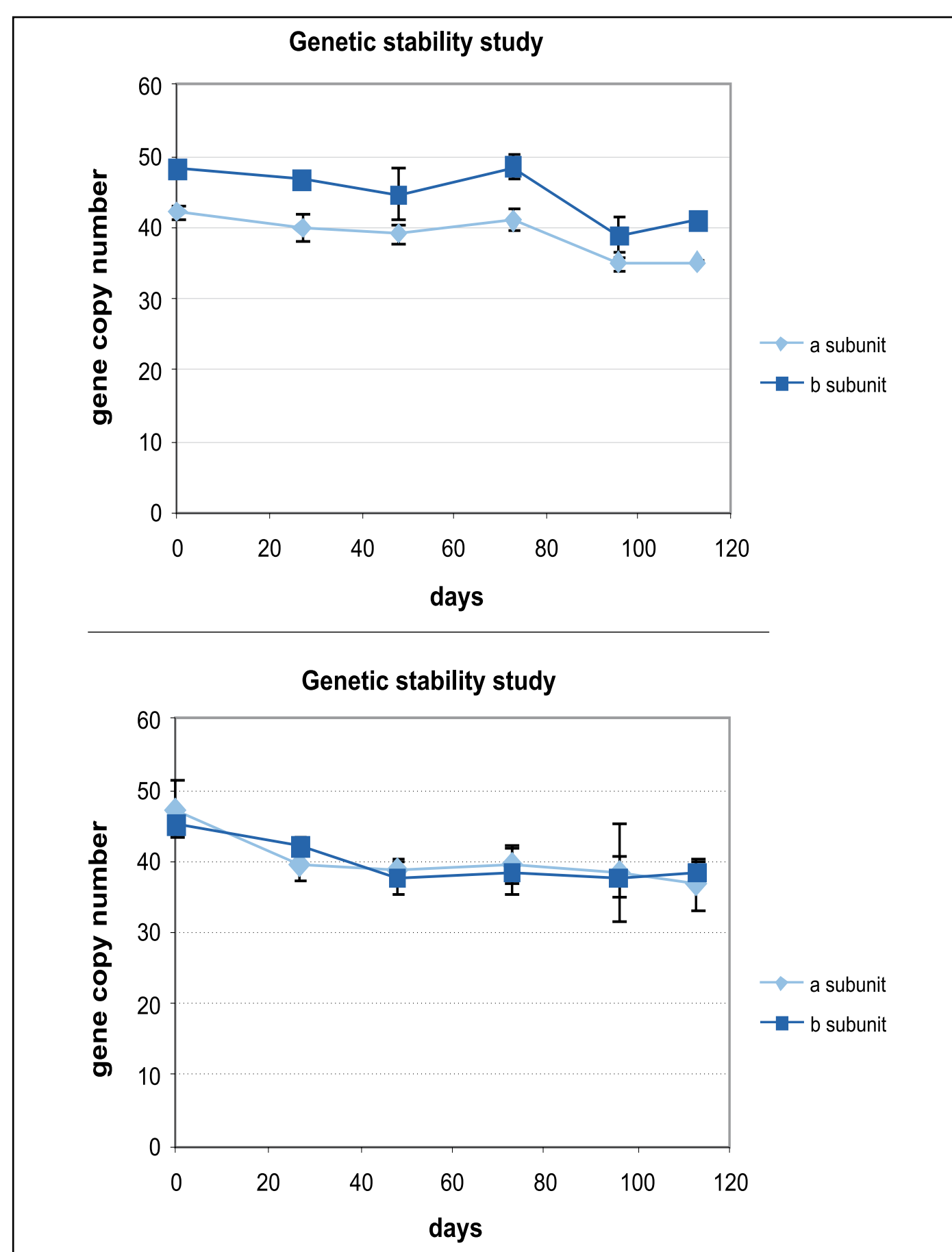
gluc and transgene copy number, separately for the A and B subunit of the recombinant protein and resistance gene.

## Results and discussion

In this study it was shown that the developed qPCR method gave promising results for transgene copy number determination for different applications during the cell line development and production process. The applied approach gave accurate and reproducible results. It is also fast, robust and easy to perform. The costs were further reduced due to the use of 10μl reactions. It was tested and applied to three different applications: genetic stability study, evaluation of transfection efficiency and testing the influence of methotrexate on transgene copy number increase.

### Genetic stability study

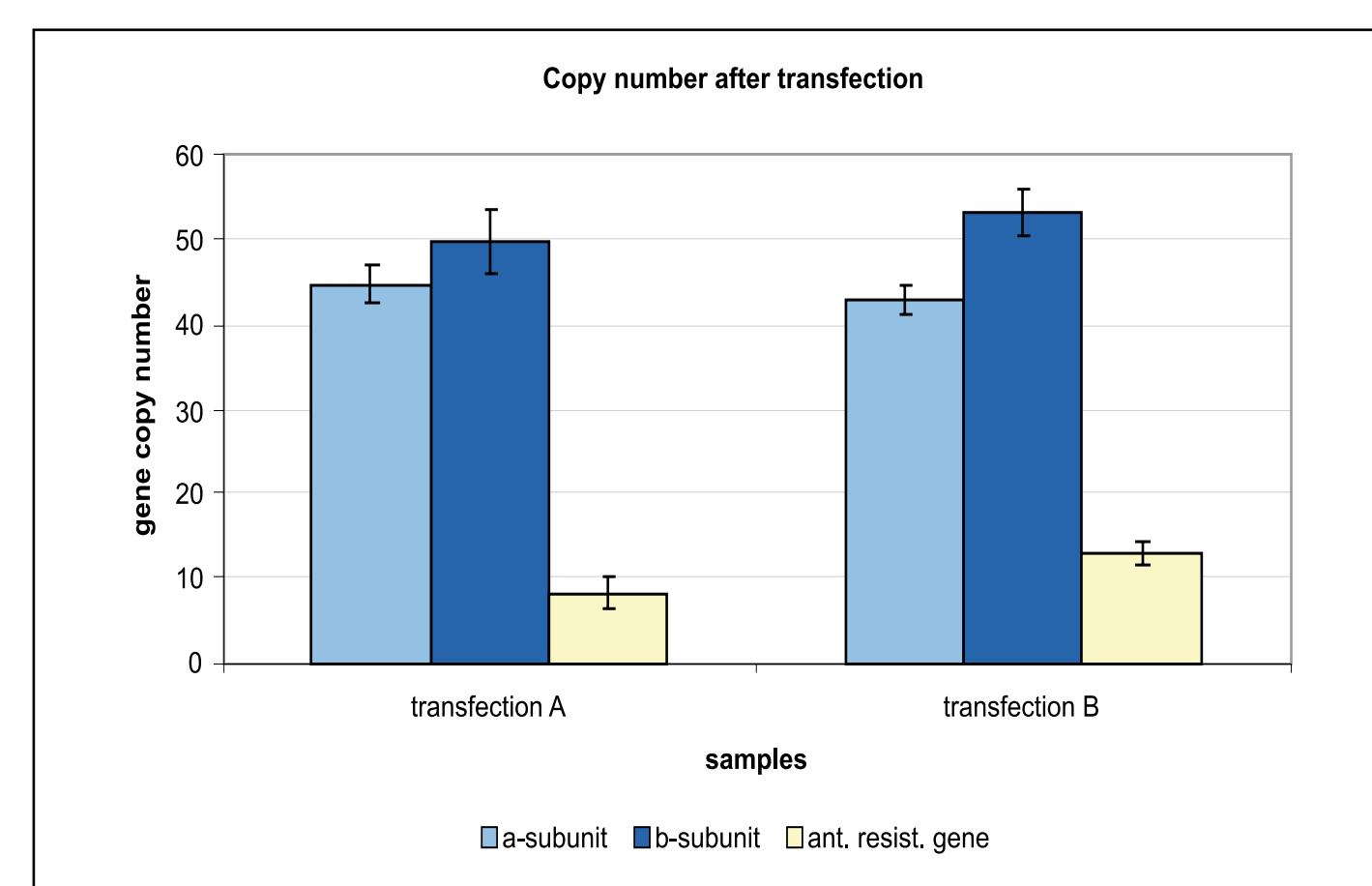
Genetic stability of clones is an important parameter in longer cultivation processes. Results of two different genetic stability tests are presented (Graph 1). A slight decrease in transgene copy number is observed, which was in the range of 15 - 20%. The difference can be assigned to the actual decrease in the copy number or to the of the method variability, which is in the range of 30%. We conclude that the evaluated clone is stable since no significant deviation in copy number is observed.



Graph 1: genetic stability tests of selected clone

### Influence of cotransfection ratio on transgenes integration

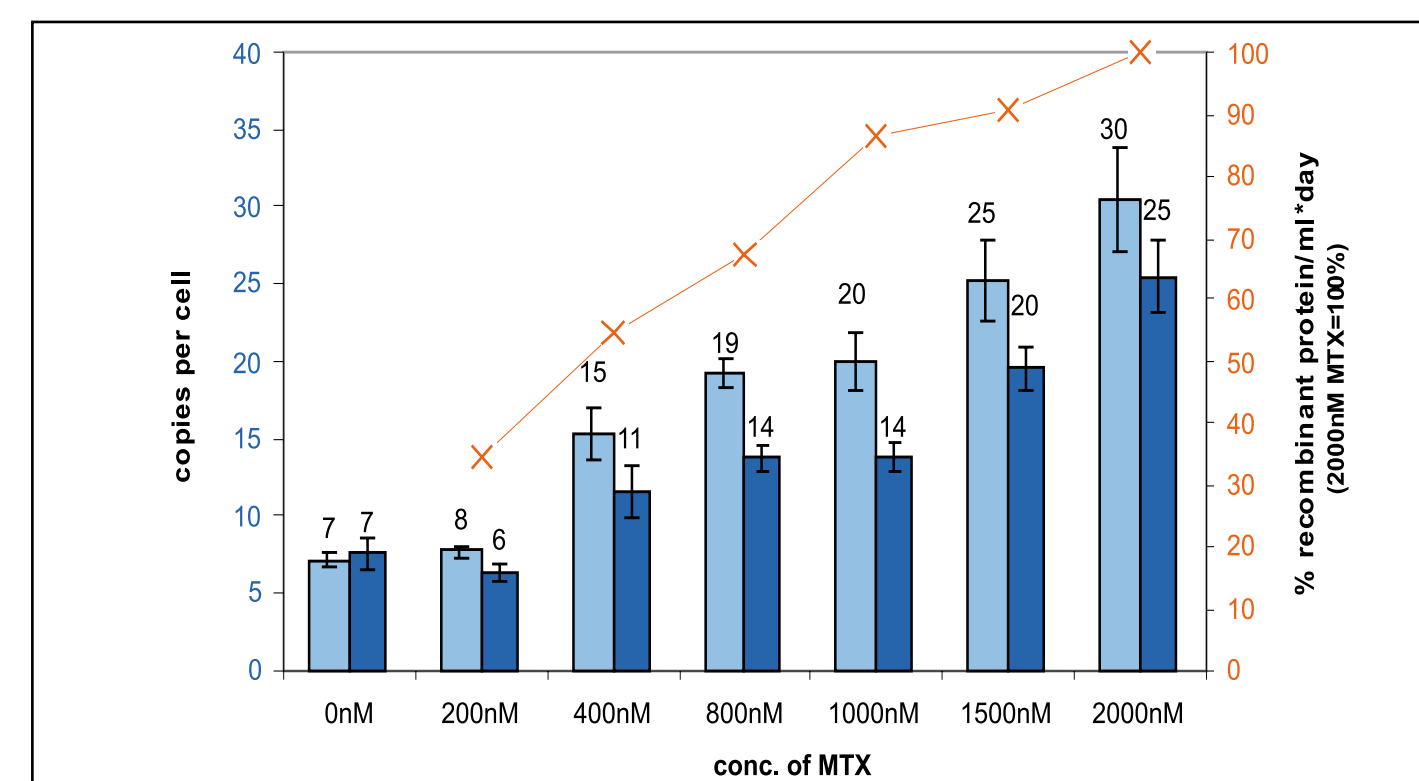
The ratio between the two plasmids used for transfection and the ratio between the copy number of both subunits of the recombinant protein and the antibiotic resistance gene was compared (Graph 2). The ratio in copy number between the recombinant protein genes and the resistance gene after integration into the chromosome was approx. 5:1 (at a transfection ratio 10:1). An appropriate ratio of 1:1 is observed between the two subunits of the recombinant protein. Since the transgenes for the subunits are on the same plasmid we can assign the slight difference to the ideal 1:1 ratio to the method variability.



Graph 2: integrated gene copy number after cotransfection with two plasmids. A and B subunit genes of recombinant protein on the plasmid and antibiotic resistance gene on another plasmid in a ratio 10:1.

### Influence of methotrexate on transgene copy number increase

CHO dhfr- cells were co-transfected with a plasmid containing the gene for DHFR and a separate plasmid containing the transgenes. Selection of cells occurred in the absence of hypoxanthine and thymidine, preventing growth of nontransformed cells. Expression of a recombinant protein can be augmented by exposing cells to methotrexate, a drug that blocks activity of DHFR. After 2-3 weeks of exposure to MTX the majority of cells die, but a small number of cells that overproduce DHFR survive. Upon MTX treatment, the transgene copy number in these cells increases (1, 2). In our case CHO dhfr- cells were grown in seven parallels with different concentrations of methotrexate. The results show, that increasing the concentration of methotrexate increased the transgene copy number per cell. In addition a larger copy number resulted in higher protein productivity (Graph 3).



Graph 3: influence of methotrexate treatment on transgenes copy number and protein production

## Conclusions

The results show that the developed qPCR method is suitable for transgene copy number determination during cell line development. The method provides accurate and reproducible results and is fast and easy to perform. It also has good prospects for partial automation which is an important aspect in industrial production. The method was successfully applied as a tool for genetic characterization of recombinant cells in biopharmaceutical industry.

## References

1. Wurm FM: **Production of recombinant protein therapeutics in cultivated mammalian cells.** Nature Biotech 2004, 22: 1393-1398
2. Butler M: **Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals.** Appl Microbiol Biotechnol 2005, 68: 283-291